Supplementary Materials for

Estrogen Receptor β Activation Inhibits Colitis by Promoting NLRP6-Mediated Autophagy

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Fig. S1. ERβ signaling alleviates IL-1β or TNF-α-induced colitis and deletion of NLRP6 drives colitis.

Fig. S2. The effects of ERβ on NLRP6 inflammasome expression and NLRP6 inflammasome assembly.

Fig. S3. The role of ERβ and NLRP6 in mitochondrial damage and autophagy during inflammation.

Fig. S4. The relationship between the ERβ-NLRP6 axis and autophagy.

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Fig. S6. NLRP6 is required for Rap-induced recovery from inflammation.

Other Supplementary Material for this manuscript includes the following:

Dataset 1. Technical data file (Table S1, Excel spreadsheet).

Dataset 2. Raw data file (Table S2, Excel spreadsheet).

Related Manuscript File. Unprocessed immunoblots (PDF file).

Movie S1. Live cell imaging to examine the co-localization of mCherry-ULK1 and EGFP-NLRP6 in starved NCM-460 cells without drug treatment.

Movie S2. Live cell imaging to examine the co-localization of mCherry-ATG16L1 and EGFP-NLRP6 in starved NCM-460 cells without drug treatment.

Movie S3. Live cell imaging to examine the co-localization of mCherry-LC3B and EGFP-NLRP6 in starved NCM-460 cells without drug treatment.

Movie S4. Live cell imaging to examine the co-localization of mCherry-BECN1 and EGFP-NLRP6 in starved NCM-460 cells without drug treatment.

Movie S5. Live cell imaging to examine the co-localization of mCherry-p62 and EGFP-NLRP6 in starved NCM-460 cells without drug treatment.

Movie S6. Live cell imaging to examine the co-localization of mCherry-PHB2 and EGFP-NLRP6 in starved NCM-460 cells without drug treatment.

Movie S7. Live cell imaging to examine the co-localization of mCherry-ULK1 and EGFP-NLRP6 in starved NCM-460 cells with DSS treatment.

Movie S8. Live cell imaging to examine the co-localization of mCherry-ATG16L1 and EGFP-NLRP6 in starved NCM-460 cells with DSS treatment.

Movie S9. Live cell imaging to examine the co-localization of mCherry-LC3B and EGFP-NLRP6 in starved NCM-460 cells with DSS treatment.

Movie S10. Live cell imaging to examine the co-localization of mCherry-BECN1 and EGFP-NLRP6 in starved NCM-460 cells with DSS treatment.

Movie S11. Live cell imaging to examine the co-localization of mCherry-p62 and EGFP-NLRP6 in starved NCM-460 cells with DSS treatment.

Movie S12. Live cell imaging to examine the co-localization of mCherry-PHB2 and EGFP-NLRP6 in starved NCM-460 cells with DSS treatment.

Movie S13. Live cell imaging to examine the co-localization of mCherry-ULK1 and EGFP-NLRP6 in starved NCM-460 cells with DSS and ERB-041 treatment.

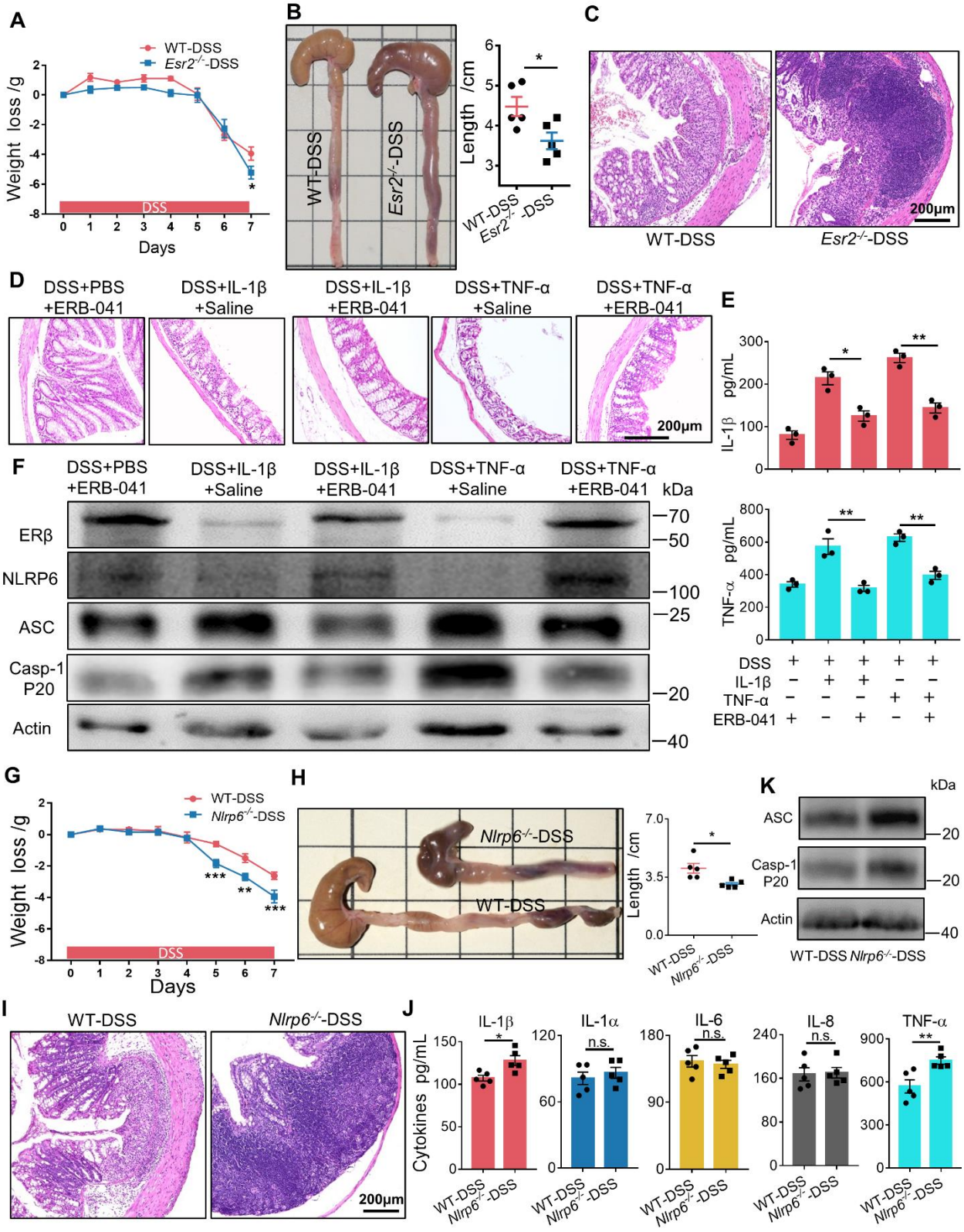
Movie S14. Live cell imaging to examine the co-localization of mCherry-ATG16L1 and EGFP-NLRP6 in starved NCM-460 cells with DSS and ERB-041 treatment.

Movie S15. Live cell imaging to examine the co-localization of mCherry-LC3B and EGFP-NLRP6 in starved NCM-460 cells with DSS and ERB-041 treatment.

Movie S16. Live cell imaging to examine the co-localization of mCherry-BECN1 and EGFP-NLRP6 in starved NCM-460 cells with DSS and ERB-041 treatment.

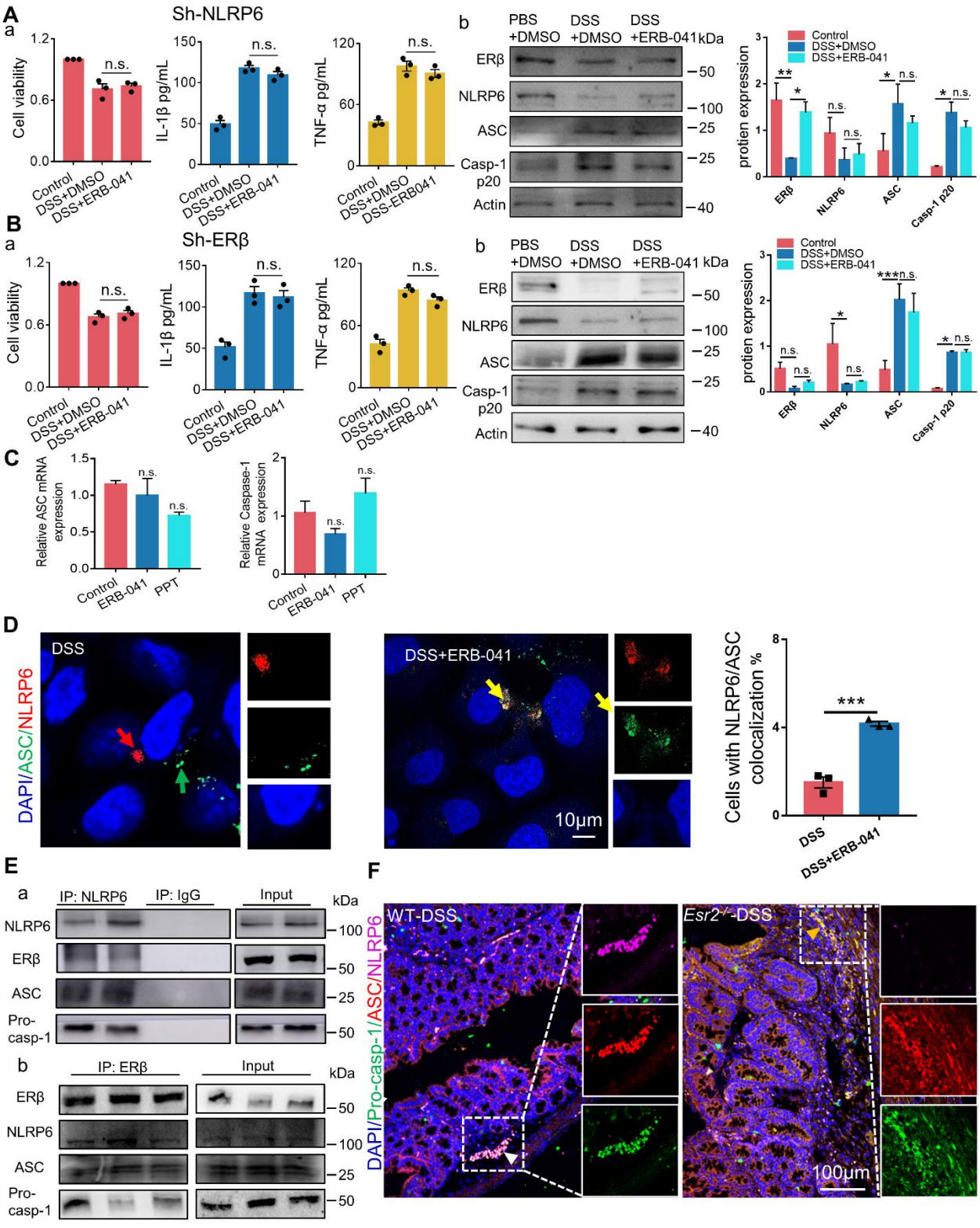
Movie S17. Live cell imaging to examine the co-localization of mCherry-p62 and EGFP-NLRP6 in starved NCM-460 cells with DSS and ERB-041 treatment.

Movie S18. Live cell imaging to examine the co-localization of mCherry-PHB2 and EGFP-NLRP6 in starved NCM-460 cells with DSS and ERB-041 treatment.


A
WT-DSS
Esr2-/--DSS
Weight loss /g
DSS
Days
B
WT-DSS
Esr2-/--DSS
Length /cm
C
WT-DSS
Esr2-/--DSS
200μm
D
DSS+PBS +ERB-041
DSS+IL-1β +Saline
DSS+IL-1β +ERB-041
DSS+TNF-α +Saline
DSS+TNF-α +ERB-041
200μm
E
IL-1β pg/mL
TNF-α pg/mL
DSS
IL-1β
TNF-α
ERB-041
F
kDa
ERβ
NLRP6
ASC
Casp-1 P20
Actin
G
WT-DSS
Nlrp6-/--DSS
Weight loss /g
DSS
Days
H
Nlrp6-/--DSS
WT-DSS
Length /cm
K
kDa
ASC
Casp-1 P20
Actin
WT-DSS Nlrp6-/--DSS
I
WT-DSS
Nlrp6-/--DSS
200μm
J
Cytokines pg/mL
IL-1β
IL-1α
IL-6
IL-8
TNF-α
n.s.

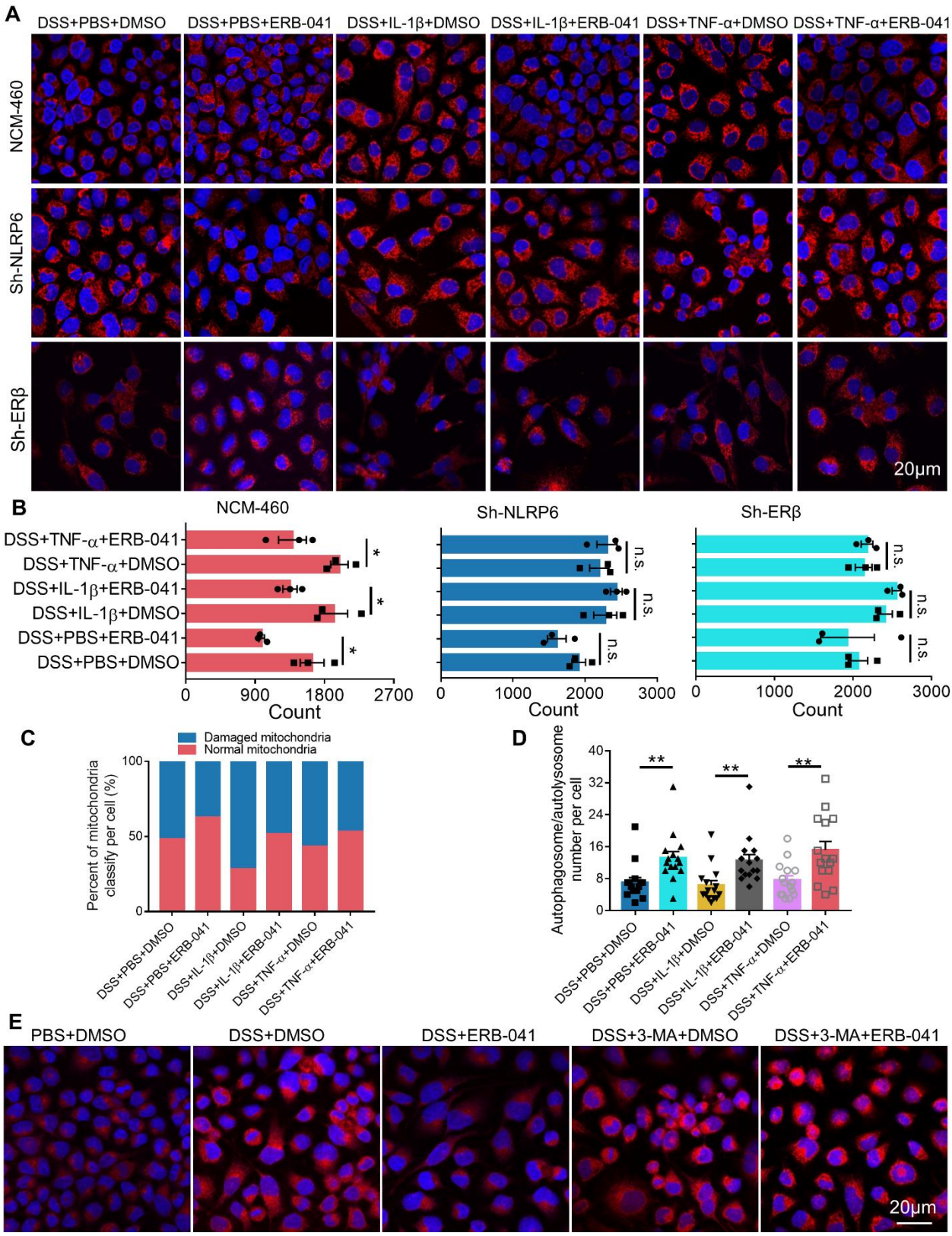
69

Fig. S1. ERβ signaling alleviates IL-1β or TNF-α-induced colitis and deletion of NLRP6 drives colitis. WT and *Esr2*$^{-/-}$ mice were administered 3% DSS in drinking water for 7 days (n = 5). (A) Weight loss of colitis model mice. Data represent mean values ± SEM. **P*< 0.05, by two-way ANOVA with Sidak's post hoc test. (B) Representative gross photographs and the colon length of different mice. Data represent mean values ± SEM. **P*< 0.05, by unpaired Student's *t*-test. (C) Representative H&E staining of distal colon sections from mice. (Scale bars, 200 μm). WT mice were administered 3% DSS in drinking water for 7 days following intraperitoneal (i.p.) IL-1β (1 μg/kg body weight) or TNF-α (500 ng/kg body weight) with(out) ERB-041 (5 mg/kg body weight) injection for 5 days (n = 3). (D) Representative H&E staining of distal colon sections. (Scale bars, 200 μm). (E) Cytokines (IL-1β and TNF-α) levels. (F) ERβ and NLRP6 inflammasome expression of mice colon. Data represent mean values ± SEM. **P*< 0.05, ***P*< 0.01, by unpaired Student's *t*-test. WT and *Nlrp6*$^{-/-}$ mice were administered 3% DSS in drinking water for 7 days (n = 5). (G) Weight loss of colitis model mice. Data represent mean values ± SEM. ***P*< 0.01, ****P*< 0.001, by two-way ANOVA with Sidak's post hoc test. (H) Representative gross photographs and quantification of the colon length of different mice. (I) Representative H&E staining of distal colon sections from mice. (Scale bars, 200 μm). (J) Colon tissue IL-1β, IL-1α, IL-6, IL-8, and TNF-α levels were measured by ELISA. (K) Inflammasome (ASC and Casp-1 p20) expression in mouse colon lysate. Data represent mean values ± SEM. **P*<0.05, ***P*< 0.01, n.s., not significant, by unpaired Student's *t*-test


A
Sh-NLRP6
a
Cell viability
IL-1β pg/mL
TNF-α pg/mL
n.s.
Control
DSS+DMSO
DSS+ERB-041
DSS-ERB041
b
PBS +DMSO
DSS +DMSO
DSS +ERB-041
kDa
ERβ
NLRP6
ASC
Casp-1 p20
Actin
protien expression
B
Sh-ERβ
C
Relative ASC mRNA expression
Relative Caspase-1 mRNA expression
ERB-041
PPT
D
DSS
DSS+ERB-041
DAPI/ASC/NLRP6
10μm
Cells with NLRP6/ASC colocalization %
E
IP: NLRP6
IP: IgG
Input
IP: ERβ
Pro-casp-1
F
WT-DSS
Esr2-/--DSS
DAPI/Pro-casp-1/ASC/NLRP6
100μm

86

Fig. S2. The effects of ERβ on NLRP6 inflammasome expression and NLRP6 inflammasome assembly. NLRP6 and ERβ knockdown NCM-460 cells (Sh-NLRP6/Sh-ERβ) were treated with 3% DSS for 24 h, then treated with ERB-041 (1 μM) for 48 h. (A-B, a). Cell viability of treated cells was detected by the CCK-8 method. Cytokines (IL-1β and TNF-α) levels in the supernatant were determined by ELISA. Each data point represents a unique experiment performed in triplicate. Data represent mean values ± SEM. n.s., not significant by unpaired Student's *t*-test. (A-B, b) Immunoblotting of treated cell lysates with specific anti-ERβ, anti-NLRP6, anti-ASC, and anti-Casp-1 p20 antibodies. Data are representative of three independent experiments. Data represent mean values ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, n.s., not significant, by two-way ANOVA with Tukey's post hoc analysis. (C) qRT-PCR assay for *ASC* and *Caspase-1* genes in the NCM-460 cells with ERB-041 or PPT stimulation. Data represent mean values ± SEM. n.s., not significant, by one-way ANOVA with Dunnett's post hoc analysis (vs. Control). (D) Representative immunofluorescence staining for NLRP6 (red), ASC (green), and DAPI nuclear stain (blue) of treated normal colon epithelial cells. (Scale bars, 10 μm). At least 400 cells were analyzed per group. Data are representative of three independent experiments. Data represent mean values ± SEM. $***P < 0.001$, by unpaired Student's *t*-test. (E-a) Co-immunoprecipitation of NLRP6 with anti-ERβ, anti-ASC or anti-Pro-casp-1 in untreated NCM-460 cells. (E-b) Co-immunoprecipitation of ERβ with anti-NLRP6, anti-ASC or anti-Pro-casp-1 in untreated NCM-460 cells. (F) Representative immunofluorescence staining for NLRP6 (violet), ASC (red), Pro-casp-1 (green), and DAPI nuclear stain (blue) in DSS-treated WT and *Esr2*$^{-/-}$ mice colon tissue. The yellow arrow represents the co-localization of Pro-casp-1 and ASC. The white arrow represents the co-localization of NLRP6, Pro-casp-1 and ASC. (Scale bars, 100 μm).


A
DSS+PBS+DMSO
DSS+PBS+ERB-041
DSS+IL-1β+DMSO
DSS+IL-1β+ERB-041
DSS+TNF-α+DMSO
DSS+TNF-α+ERB-041
NCM-460
Sh-NLRP6
Sh-ERβ
20μm
B
NCM-460
Sh-NLRP6
Sh-ERβ
DSS+TNF-α+ERB-041
DSS+TNF-α+DMSO
DSS+IL-1β+ERB-041
DSS+IL-1β+DMSO
DSS+PBS+ERB-041
DSS+PBS+DMSO
*
n.s.
0
900
1800
2700
1000
2000
3000
Count
C
Damaged mitochondria
Normal mitochondria
Percent of mitochondria classify per cell (%)
100
50
0
D
Autophagosome/autolysosome number per cell
40
32
24
16
8
0
**
E
PBS+DMSO
DSS+DMSO
DSS+ERB-041
DSS+3-MA+DMSO
DSS+3-MA+ERB-041
20μm

105

Fig. S3. The role of ERβ and NLRP6 in mitochondrial damage and autophagy during inflammation. NCM-460 cells, NLRP6 and ERβ knockdown cells (Sh-NLRP6/Sh-ERβ) were stimulated with 3% DSS for 24 h, then IL-1β (10 ng/mL) or TNF-α (5 ng/mL) with(out) ERB-041 (1 μM) treated for 48 h. After treatment, cells were incubated with 100 nM MitoTracker Red CMXRos for 30 min to trace morphological changes in mitochondria. (A) The samples were observed by fluorescence microscopy. (Scale bars, 20 μm). (B) Histograms quantification of ROS positive cells indifferent groups. Each data point represents a unique experiment performed in triplicate. Data represent mean values ± SEM. **P*< 0.05, n.s., not significant, by unpaired Student's *t*-test. (C) Mitochondrial quantitative analysis of mitochondria morphology in different treated NCM-460 cells. (Normal: cristae are maintained. Damaged: mildly swollen and increased mitochondria fission or fusion or severely swollen and >70% of cristae are missing, or highly dysmorphic and electron dense). (D) Autophagosome and autolysosome quantification in the treated NCM-460 cells. At least 15 cells in each group were analyzed. Experiment performed in triplicate. Data represent mean values ±SEM. ***P*< 0.01, by unpaired Student's *t*-test. (E) NCM-460 cells were stimulated with 3% DSS for 24 h, then treated with 3-MA together with or without ERB-041 (1 μM) for 48 h. Different treated cells were incubated with 100 nM MitoTracker Red CMXRos for 30 min to trace morphological changes in mitochondria. The samples were observed by fluorescence microscopy. (Scale bars, 20 μm).

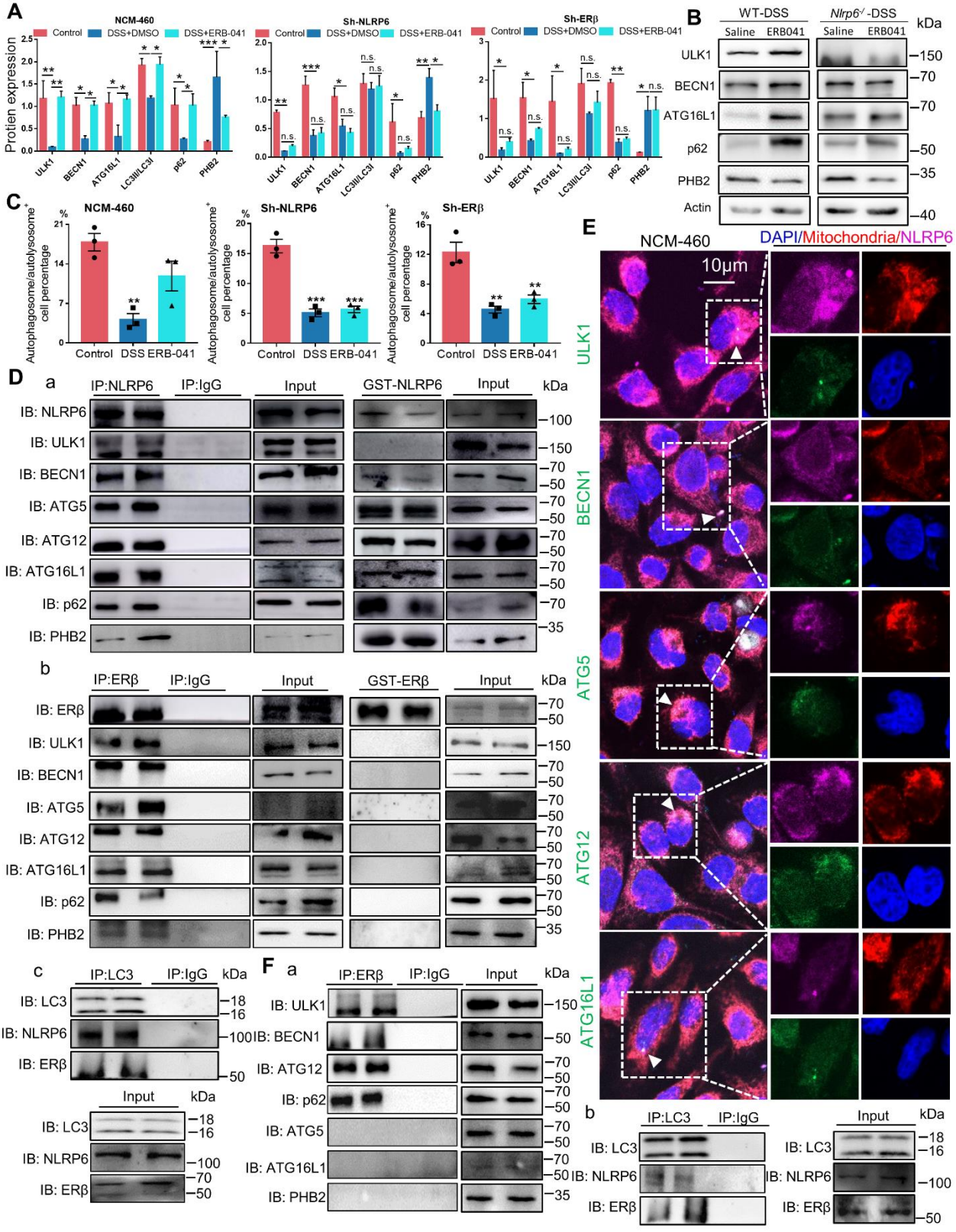
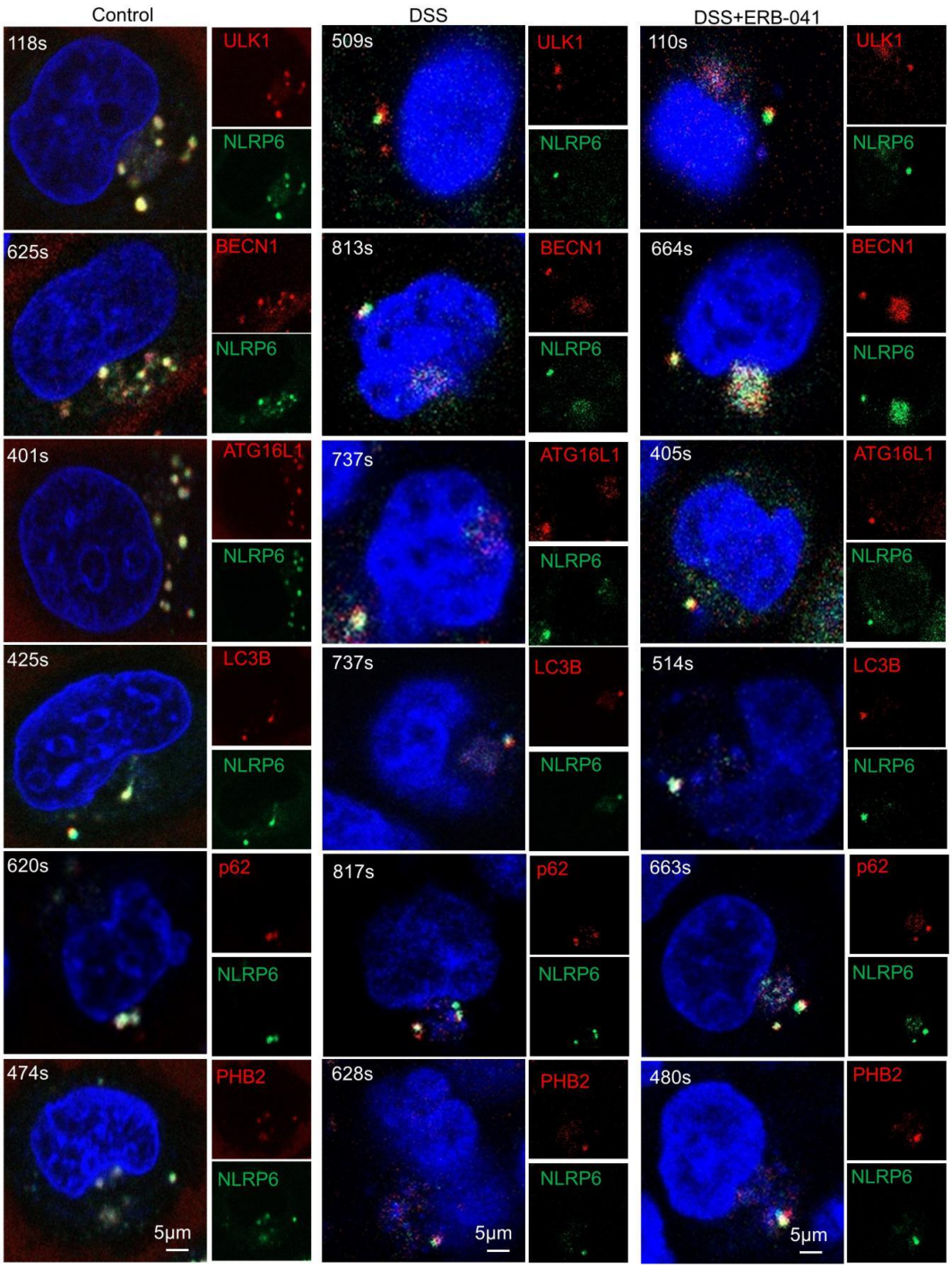
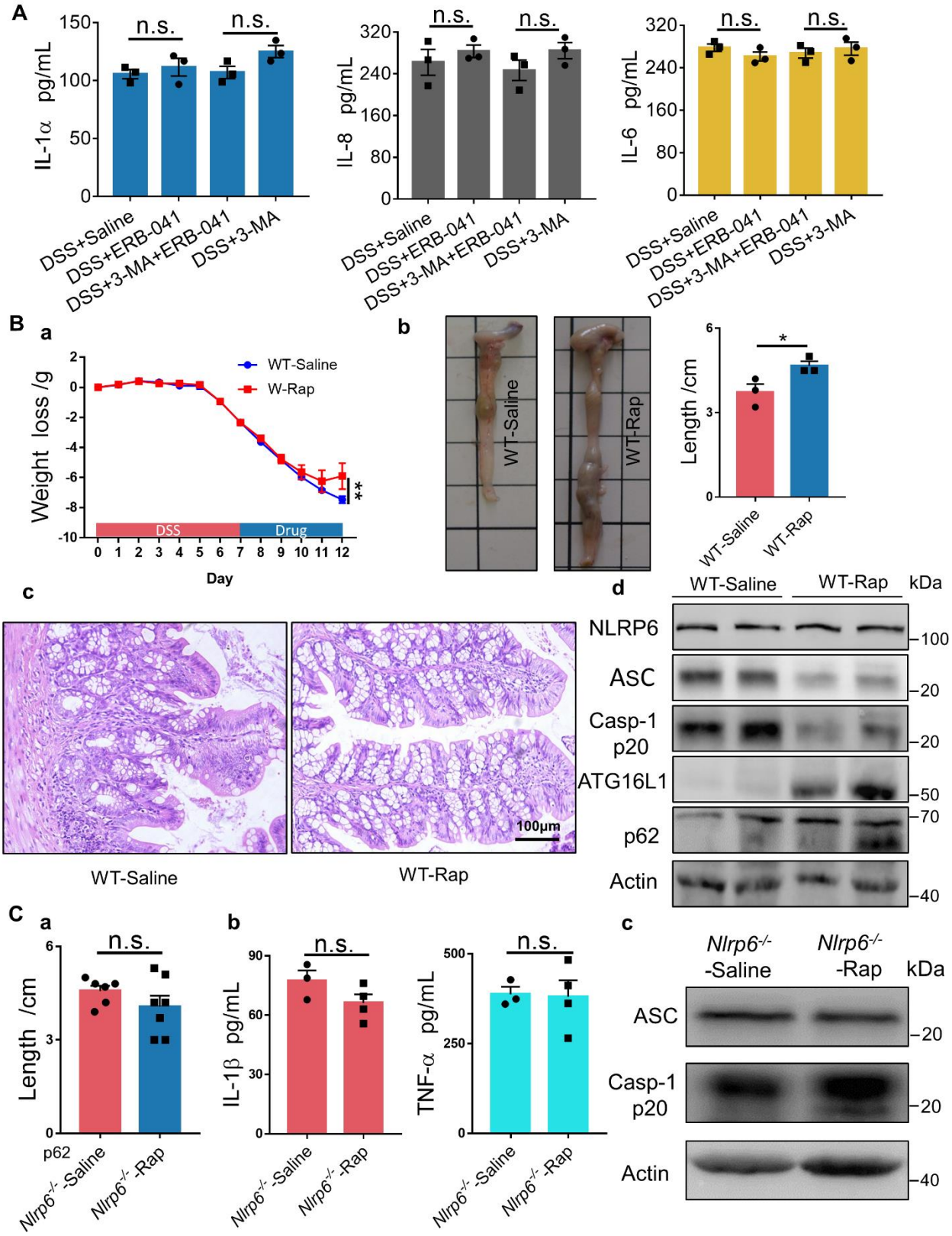

A
NCM-460
Sh-NLRP6
Sh-ERβ
Control
DSS+DMSO
DSS+ERB-041
Protien expression
ULK1
BECN1
ATG16L1
LC3II/LC3I
p62
PHB2
B
WT-DSS
Nlrp6-/--DSS
Saline
ERB041
kDa
ULK1
BECN1
ATG16L1
p62
PHB2
Actin
C
NCM-460
Sh-NLRP6
Sh-ERβ
Autophagosome/autolysosome cell percentage
Control
DSS
ERB-041
D
a
IP:NLRP6
IP:IgG
Input
GST-NLRP6
IB: NLRP6
IB: ULK1
IB: BECN1
IB: ATG5
IB: ATG12
IB: ATG16L1
IB: p62
IB: PHB2
b
IP:ERβ
GST-ERβ
IB: ERβ
c
IP:LC3
IB: LC3
E
NCM-460
DAPI/Mitochondria/NLRP6
10μm
ULK1
BECN1
ATG5
ATG12
ATG16L1
F
a
b

Fig. S4. The relationship between the ERβ-NLRP6 axis and autophagy. NCM-460 cells, NLRP6 knockdown (Sh-NLRP6), and ERβ knockdown (Sh-ERβ) NCM-460 cells were stimulated with 3% DSS for 24 h, then treated with ERB-041 (1 μM) for 48 h. (A) Western blot analysis of autophagy protein. Each data point represents a unique experiment performed in triplicate. Data represent mean values ± SEM. **P*< 0.05, ***P*< 0.01, ****P*< 0.001, n.s., not significant, by two-way ANOVA with Tukey's post hoc analysis. (B) Western blot analysis of colon tissue from WT and *Nlrp6*$^{-/-}$ mice treated with 3% DSS followed by saline or ERB-041. Total lysates were prepared, and western blots were probed with specific anti-ULK1, anti-BECN1, anti-ATG16L1, anti-p62, and anti-PHB2 antibodies. (C) Confocal microscopy of EGFP-mCherry-LC3B (yellow) expressed in different treated NCM-460 cells. Changes in cells containing autophagy puncta were calculated from at least 10 areas (400 cells) of interest pooled from three independent experiments. Data represent mean values ± SEM. ***P*<0.01, ****P*<0.001, by one-way ANOVA with Dunnett's post hoc analysis (vs. Control). (D-a) Co-immunoprecipitation of autophagy protein with NLRP6 in untreated NCM-460 cells and i*n vitro* interaction of purified GST-NLRP6 with autophagy proteins. (D-b) Co-immunoprecipitation of autophagy protein with ERβ in untreated NCM-460 cells and i*n vitro* interaction of purified GST-ERβ with autophagy proteins. (D-c) Co-immunoprecipitation of NLRP6 and ERβ with LC3 in untreated NCM-460 cells. (E) Representative images showing co-localization of endogenous NLRP6 (Violet) and ULK1/BECN1/ATG5/ATG12/ATG16L1 (green) on mitochondria (red) in untreated NCM-460 cells. White arrow indicates the co-localization of endogenous NLRP6 and autophagy protein on mitochondria. (Scale bars, 10 μm). (F-a) Co-immunoprecipitation of autophagy protein with ERβ in untreated HEK293T cells. (F-b) Co-immunoprecipitation of NLRP6 and ERβ with LC3 in untreated HEK293T cells.



141

142 **Fig. S5. The effects ERB-041 on NLRP6 localization to autophagy machinery.** NCM-460 cells were stimulated
143 with 3% DSS for 24 h, then treated with ERB-041 (1 μM) for 48 h. Live NCM-460 cells stably expressing EGFP-
144 NLRP6 and mCherry-ULK1/BECN1/ATG16L1/LC3B/p62/PHB2 were starved of amino acids in Hank's Balanced
145 Salt Solution (HBSS) for one hour and then imaged continuously for 15 minutes. Co-localization images are shown.
146 See supplementary videos for live cell recording.


A
IL-1α pg/mL
IL-8 pg/mL
IL-6 pg/mL
n.s.
DSS+Saline
DSS+ERB-041
DSS+3-MA+ERB-041
DSS+3-MA
B
a
Weight loss /g
WT-Saline
W-Rap
DSS
Drug
Day
b
WT-Saline
WT-Rap
Length /cm
c
100μm
WT-Saline
WT-Rap
d
WT-Saline
WT-Rap
kDa
NLRP6
ASC
Casp-1 p20
ATG16L1
p62
Actin
C
a
Length /cm
p62
Nlrp6-/- -Saline
Nlrp6-/- -Rap
b
IL-1β pg/mL
TNF-α pg/mL
c
Nlrp6-/- -Saline
Nlrp6-/- -Rap
ASC
Casp-1 p20
Actin

147

Fig. S6. NLRP6 is required for Rap-induced recovery from inflammation. WT mice were administered 3% DSS in drinking water for 7 days followed by intraperitoneal (i.p.) injection of saline or the ERB-041 (5 mg/kg body weight) with or without 3-MA (1.5 mg/kg body weight) for 5 days (n = 5). (A) IL-1α, IL-8, and IL-6 levels were measured by ELISA. Data represent mean values ± SEM. n.s., not significant, by Unpaired Student's *t*-test. (B) WT mice were administered 3% DSS in drinking water for 7 days followed by intraperitoneal (i.p.) injection of saline or rapamycin (Rap) (2 mg/kg body weight, n = 3) for 5 days. (a) Weight loss, (b) representative gross photographs and the colon length of different mice, (c) representative H&E staining of distal colon sections from mice (scale bars, 100 μm), and (d) expression levels of NLRP6 inflammasome (NLRP6, ASC, and Casp-1 p20) and autophagy protein (ATG16L1, and p62) were analyzed. Data represent mean values ±SEM. **P*< 0.05, ***P*< 0.01, by two-way ANOVA with Sidak's post hoc test or unpaired Student's *t*-test. (C) *Nlrp6*$^{-/-}$ mice were administered 3% DSS in drinking water for 7 days followed by intraperitoneal (i.p.) injection of saline (n = 6) or rapamycin (Rap) (2 mg/kg body weight, n = 7) for 5 days. (a) Colon length, (b) cytokines, and (c) inflammasome (ASC, and Casp-1 p20) expression was analyzed. Data represent mean values ± SEM. n.s., not significant, by unpaired Student's *t*-test.